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Identification of Immunogens of ´Mycoplasma pneumoniae´ by Protein Blotting

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IDENTIFICATION OF IMMUNOGENS OF MYCOPLASMA PNEUMONIAE BY PROTEIN BLOTTING

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SUMMARY

Proteins of Mycoplasma pneumoniae were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose sheet by blotting. Sera obtained from infected hamsters and immunized rabbits were then incubated with the nitrocellulose strips. Proteins which are capable of eliciting antibodies were detected by indirect immunoradioautography using ^{125}I-labeled antisera against hamster or rabbit IgG. Antibodies to seven immunogens were demonstrated in the sera of hamsters infected with M. pneumoniae by inhalation, while many more proteins were found to be capable of stimulating antibodies in rabbits immunized parenterally with mycoplasmas. This method should prove useful for identifying those components of a microorganism which elicit antibody responses and contribute to the protective state.

1. INTRODUCTION

Mycoplasma pneumoniae is one of the most common pathogens of human respiratory disease. Illnesses associated with M. pneumoniae infection range from a mild upper respiratory infection and tracheobronchitis to acute pneumonia (1,2,3,4). Attempts have been made to develop an effective vaccine against M. pneumoniae in different laboratories using live or killed whole organisms (5,6,7,8,9). We reported earlier the identification of external proteins of M. pneumoniae, including a protein possibly involved in the attachment of mycoplasma organisms to the respiratory epithelium (10). However, the capability of individual mycoplasma

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proteins to elicit antibodies during a natural infection has not been reported previously.

The identification of those components of a microorganism which elicit antibody production during the course of an infection would contribute to a better understanding of the pathogenesis of disease, and the host's protective mechanisms, i.e., immune response. In addition, identification and isolation of the specific immunogens responsible for protective immunity may lead to the development of effective vaccines without the adverse side effects associated with administration of whole organisms.

2. MATERIALS AND METHODS

2.1 Preparation of Mycoplasmas

M. pneumoniae strain, M-129, (ATCC No. 29342) was originally isolated from a patient with pneumonia. The low passages (12-15 broth passages) of this organism retained virulence, judged by their ability to produce pneumonia in golden Syrian hamsters and were used in the present study. Cultures were maintained in Hayflick's medium supplemented with 20% agammaglobulin horse serum, 10% yeast dialysate, and penicillin (1,000 U/ml) (10).

Monolayer cultures were grown in 32 oz. acid-cleaned prescription bottles as previously described (11). After incubation at 37°C for 36-48 hrs. in 5% CO_2 with air, the medium was decanted, and the organisms were washed five times with phosphate-buffered saline (PBS), pH 7.2. Organisms and the pelleted organisms (12,000 xg, 15 min.) were processed for sodium dodecyl sulfate (SDS) slab gel electrophoresis.

2.2 Preparation of Antisera

Hamsters were infected by inhalation of viable M. pneumoniae aerosols as described previously (12). Four ml of mycoplasma organisms (~7.5 x 10^8 CFU/ml) were used to generate the aerosol for each exposure. Sera were collected from individual animals 8 wks. after the infection. Hyperimmunized rabbit serum was a gift from Dr. Wallace A. Clyde, Jr.

2.3 Gel Electrophoresis

Discontinuous SDS-gel electrophoresis in 10% polyacrylamide gel was performed as previously described (10), except that a slab gel electrophoresis cell was used (Model 220, Bio-Rad). The initial current of 10 mA/gel was increased to 25 mA/gel after the tracking dye migrated into the separating gel. Total time for electrophoresis was about 4 hrs. for 9-cm gels. Molecular weights of the proteins were determined by co-electrophoresis of known molecular weight standards.

2.4 Method of Transfer

The transfer of the protein bands from the SDS gel onto the nitrocellulose sheets was achieved using a slight modification of the technique described by Bowen et al. (13). After electrophoresis, the slab gel (for example, 9 x 14/cm^2) was immersed in 100 ml of transfer buffer (10 mM tris-HCl, pH 7.4, 50 mM NaCl, 2 mM Na-EDTA, 0.1 mM dithiothreitol) containing 4M urea, and gently agitated for 3 hours. This treatment removes SDS (14) and facilitates subsequent renaturation of the proteins (15). After the first hour of incubation, the initial buffer was replaced with fresh buffer. After this step, a 1-cm wide strip was cut off from the gel and stained with Coomassie Blue (10), and the remaining gel was sandwiched between two sheets of unmodified nitrocellulose (0.45 µm, Millipore). The "sandwich" apparatus was the same as that described by Bowen et al. (13), except that DNA blotting pads (BRL) were substituted for the foam pads and two plexiglas plates with numerous holes were used to give additional mechanical support when the apparatus was assembled. The sandwich was incubated in 2 l of transfer buffer for 36 to 48 hrs. at 37°C. The buffer was replaced after 12 hrs. with fresh buffer.

After protein transfer, the nitrocellulose sheet was removed from the sandwich and cut into strips 5 mm wide. One strip was stained with 0.1% aniline blue in 45% methanol, 10% acetic acid, and 45% H_2O for 30 min. and destained for 1 hr. in 3 changes of 90% methanol, 2% acetic acid, and 8% water as previously described (13). The rest of the nitrocellulose strips were transferred to individual plastic tissue culture tubes (16 x 150 mm, Falcon).

Five ml of NET-NP40/AHS buffer, pH 7.4 (50 mM Tris-HCl, 150 mM NaCl, 5 mM Na-EDTA, 0.25% gelatin, and 2% agamma horse serum) were added to each tube and the tubes were gently rocked for 30 min. After this treatment, the NET-NP40/AHS buffer was removed and the strips were stored at -80°C, or immediately used for the identifcation of immunogens.

2.5 Identification of Immunogens of Mycoplasma pneumoniae

The identification of immunogens of M. pneumoniae was carried out sequentially by: (1) soaking each strip in 5 ml of NET-NP40/AHS buffer containing 0.01% sodium azide and 25 µl of antiserum from experimentally infected hamsters or from immunised rabbits for 6 to 12 hrs. at room temperature with gentle rocking, (2) washing each strip in 4 changes of NET-NP40/AHS for 1 hr., and (3) incubating with 10 to 25 µl of ^{125}I-labeled IgG fraction of rabbit anti-hamster IgG or with goat anti-rabbit IgG for 6 to 12 hrs. ^{125}I-labeled immunoglobulins were prepared by the chloramine-T method (16). The strips were then extensively washed with several changes of PBS for 3 hrs. and dried with a heating lamp. Radioautographs were produced with Xomat film (Kodak) at -80°C for 12 to 24 hrs. with intensifying screen (Dupont).

3. RESULTS AND DISCUSSION

When proteins of M. pneumoniae separated on SDS-slab gel were transferred to nitrocellulose and renatured, it was possible to identify individual protein immunogens of this organism by indirect immunoradioautography using serum from experimentally infected animals. Proteins

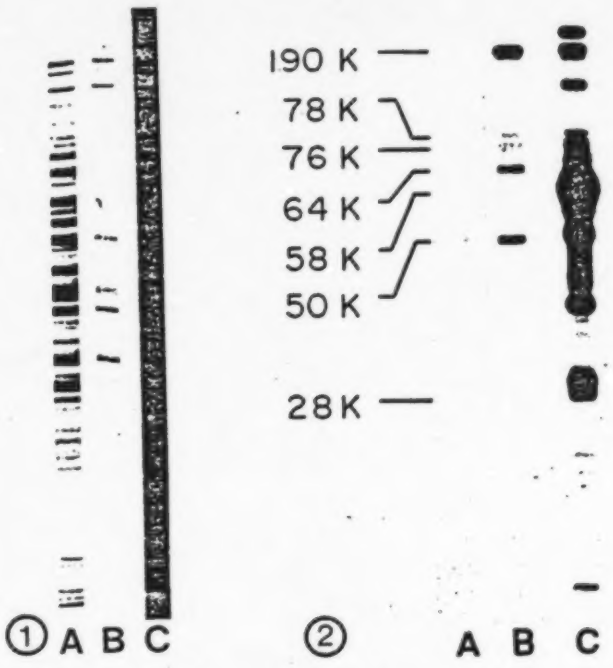

Figure 1 (Left). Transfer of proteins of M. pneumoniae from SDS polyacrylamide gel (A) to nitrocellulose sheet (C). Some residual proteins are retained in the gel (B) after the transfer. A and B stained with 0.025% coomassie blue (10), and C stained with 0.1% aniline blue (13).

Figure 2 (Right). Detection of immunogens of M. pneumoniae on nitrocellulose blots by immunoradioautography. (A) incubated with preimmune hamster serum, (B) incubated with serum from hamsters infected with M. pneumoniae by inhalation, (C) incubated with serum from rabbits hyperimmunized with M. pneumoniae organisms. Molecular weights were estimated by co-electrophoresis with proteins of known molecular weights (BioRad).

of all different molecular weights were transferred from the SDS-gel (Figure 1a) to the nitrocellulose sheet (Figure 1c) with good fidelity, although some individual protein bands were not transferred quantitatively as indicated by some stained residues remaining in the gel after transfer (Figure 1b). Renaut et al. (17) indicated that the percent efficiency of transferring proteins to diazobenzyloxymethyl cellulose was reciprocally proportional to their molecular weights. More recently, Burnette (18) also reported that the time required to

transfer proteins from polyacrylamide gels to nitroclelulose sheets by electrophoresis is directly proportional to their molecular weights. However, in the present study, the transfer efficiency did not appear to depend solely upon the molecular weight. Although the amount of residues retained in the gel varied from band to band, there is no pattern of transfer that could be attributed to the molecular weight alone. Unlike the studies previously mentioned which dealt with soluble proteins, the gels in this study were prepared with proteins from whole organisms, including many hydrophobic membrane proteins. It is conceivable that the transfer efficiency of individual proteins also depends on physico-chemical characteristics other than molecular weight. For instance, the dielectric constant of a protein will determine its mobility across the minute distance in buffer to the nitrocellulose sheet. Another factor influencing retention of proteins by the gel is the binding capacity of the nitrocellulose sheet for proteins. Even for the most soluble proteins, once a particular site on the nitrocellulose sheet is saturated, the excess protein will tend to be retained in the gel.

For the present study, the lower transfer efficiency of some proteins did not interfere with the detection of immunogens by specific antibodies because the binding of antibody to antigen on the nitrocellulose strip is dependent upon their specific interaction, which requires only minute quantities of antigen. This high sensitivity has been demonstrated by Burnette (18) who showed that as little as 1-2 ng of a specific antigen can be detected on a nitrocellulose blot with a hyperimmune serum.

Figure 2 shows the indirect radioautographic detection of M. pneumoniae immunogens on nitrocellulose blots following protein transfer from a SDS-polyacrylamide gel. Seven immunogens were detected by incubation with serum from hamsters experimentally infected with M. pneumoniae aerosols (Figure 2b). The faint band which appears on the nitrocellulose blot incubated with preimmune hamster serum is probably due to a nonspecific

binding by ^{125}I-labeled antihamster serum to the agamma horse serum-soaked nitrocellulose blot, since omission of the hamster serum gave the same single weak band (data not shown). A question raised during this study was whether the seven reacting bands were the only antigenically reactive sites (epitopes) renatured to their "natural" conformation and thus capable of reacting with antibody, since the epitopes on the fractionated protein molecules could be irreversibly denatured by the treatment of SDS. Although it is generally not a problem when polyvalent antisera are used for detection (18), a partial answer is provided by parallel tests using hyperimmunized rabbit serum, as shown in Figure 2c, in which at least 20 bands were demonstrated. This indicates that proteins of a wide range of molecular weights with restored antigenicity were present to react with available antibody.

Accordingly, the difference shown between Figures 2b and 2c may be due to the antibody idiotypes produced by different methods of exposure to the antigens: the hamster response was related to experimental respiratory tract infection, whereas the rabbits were exposed parenterally to whole organisms in combination with complete adjuvant. The outcome of immunization is dependent upon route and method of exposure of the host antigens. For example, Kaltreider (19) showed that immune responses in dogs to a complex antigen (sheep erythrocytes) was different when the antigen was administered by different routes.

Since M. penumoniae is a surface parasite of the respiratory tract, results obtained by hamster infection probably have more relevance to natural disease and may reflect those components of antibody stimulation which are important in terminating infection and in constituting protective immunity. Many earlier studies (20,21,22) of the antigenic determinants of M. pneumoniae by serologic reactions have focused on the glycolipid hapten which is involved in the complement fixation test often used for clinical diagnosis (23). More attention needs to be given to the protein

antigens, particularly those on the mycoplasma surface, such as the components related to organism attachment to host cells. The methods described in this report may facilitate such studies. Definition of mycoplasma antigens which elicit a protective host immune resonse can provide clues for future development of improved vaccines.

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